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13. SUPPLEMENTARY NOTES

14. ABSTRACT
The primary objective of this project is to determine if extracorporeal shock wave (ESW)-stimulated periosteum improves cartilage repair when it is used as an autograft to fill a defect in the articular surface of goats. A miniature fiber optic pressure sensor will be inserted into the tibial periosteum of 6 animals to measure the actual shock waveform in the tissue for two ESW doses (energy densities). In 12 goats, tibial periosteum stimulated by one of the 2 doses of ESWs (n=6) will be harvested, 4 days post-treatment, as an autograft for implantation into one 1 cm2 defect surgically produced in the trochlear groove of the knee joint of the same goat. Non-ESW-treated periosteum will serve as the control group (n=6). All animals will be sacrificed after 16 weeks, and the reparative tissue will be quantified histomorphometrically by determining the areal percentage of selected tissues in the original cartilage defect area.

15. SUBJECT TERMS
Shock waves, cartilage, goat

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I. INTRODUCTION

The primary objective of this project is to determine the conditions under which extracorporeal shock wave (ESW)-stimulated periosteum improves cartilage repair when it is used as an autograft to fill a defect in the articular surface of goats. Periosteum, which contains cells with chondrogenic potential, has been investigated as an autograft for cartilage repair procedures. However, this approach is limited because the cambium layer of the periosteum is normally only 2-5 cells thick, and some of these cells are lost during the harvest procedure. We have previously demonstrated that extracorporeal shock waves, at doses approved by FDA for treatment of certain disorders, can stimulate up to a 10-fold increase in the thickness of rat and rabbit periosteum after only 4 days.

This protocol deals with the therapeutic use of shock waves. Shock waves are pressure waves of very short duration (a few microseconds). The initial peak compressive wave is followed by a lower amplitude tensile wave. The ESWs can be produced by apparatus that focus the waves at a certain location in the body or produce waves which radiate from the shock wave head. Normally treatments apply up to 3000 shocks in a session. The "dose" of shock waves is measured in energy density. For our studies the energy densities to be used will be: 0.15 mJ/mm2 ("low") and 0.45 mJ/mm2 ("high"). This range is approved by FDA for other indications than the one we will be investigating, but it demonstrates that shock waves in this dose range have an acceptable safety profile.

One of the principal delays in our performing the DOD study relates to the shock wave apparatus being employed. In our prior rat and rabbit studies, we employed a commercially-available, focused shock wave apparatus on loan from the manufacturer, which employed the detonation of a spark to create the shock wave which was focused by a parabolic reflector. That apparatus was subsequently taken out of service by the manufacturer. According to the literature, similar results should have been expected from: 1) an unfocused (radial) shock wave apparatus; and 2) a focused apparatus employing piezoelectric crystals to create the focused shock wave. During the initial period of this DOD project we conducted pilot experiments, using goats on other studies, with these radial and piezoelectric apparatus to assess their effects in stimulating periosteal proliferation in goats after 4 days. We did not find the expected increase in the cell number and thickness of periosteum, as we found in the rat and rabbit studies using the initial shock wave apparatus. Therefore, we decided to obtain the newer version of the original focused shock wave apparatus. We have now been conducting in vitro experiments with the focused shock wave apparatus from the original manufacturer to insure that its shock wave waveform is capable of stimulating the proliferation of periosteum-like cells prior to completing the goat experiment.

During this prior project period we investigated the effects of shock waves from the focused ESW apparatus on mesenchymal stem cells in vitro. Our goal was to begin to determine the mechanism by which ESWs stimulate a proliferation of osteogenic and chondrogenic cells and to determine the effects of ESWs on the differentiation potential of the cells. The differentiation experiments were performed in monolayer and in a three-dimensional hydrogel to serve as a "tissue simulant." Our hypothesis was that cells exposed to ESWs released a paracrine factor that stimulated cell proliferation.

II. BODY

Following are the tasks comprising the Statement of Work and our related achievements

Specific Aim #1

Task 1. Measurement of the ESW pressure waveform in the periosteum of goats

1.a. Measure the pressure waveforms for select settings of the ESW apparatus in a water bath, for specific locations of sensor away from the head of the ESW device.

1.b. Insert a pressure sensor into the periosteum of the right proximal tibial of6 goats to measure the pressure waveform in the periosteum for select settings of the ESW apparatus.

The data related to these tasks were documented in the Annual Report of December, 2011

Specific Aim #2usi

Task 2. Histological evaluation of the thickness and number and type of cells in the ESW-stimulated periosteum and controls

2.a. Histological processing of periosteum from 12 goats which underwent ESW treatment and from 6 sham-treated goats

2.b. Histomorphometric evaluation of the thickness of the periosteum and number and type of cells making up the periosteum

This task is to be completed in the upcoming project period.

Task 3. Determination of the chondrogenic potential of ESW-stimulated cambium cells *in vitro*

3.a. Isolate cells from the enzymatically-digested ESW-stimulated periosteum and sham-treated controls, 4 days post-ESW treatment, and grow in monolayer

3.b. Produce pellet cultures of the periosteal cells in chondrogenic medium

3.c. Process pellets for histological evaluation

3.d. Histomorphometric evaluation of the pellet

We have implemented cultures of mesenchymal stem cells (MSCs) in suspension to enable us to determine the effects of ESWs *in vitro* on cell proliferation and differentiation.

Materials and Methods

Bone Marrow-Derived Mesenchymal Stem Cell Isolation

Bone marrow aspirates were collected from the iliac crest of adult Spanish goats. Cells were suspended in "expansion medium" [DMEM-LG containing 10% fetal bovine serum (FBS; Invitrogen), 1% penicillin/streptomycin (Invitrogen), with the supplement of 10 ng/ml recombinant human fibroblast growth factor-2 (FGF-2; R&D Systems)], and then expanded in a monolayer flask at 37C. Second passage cells at 80-90% confluence were collected to be used for different groups of the experiments.

Gelatin (Gtn) Hydroxyphenyl Propionic Acid (HPA) Hydrogel

A gelatin (Gtn) hydroxyphenyl propionic acid (HPA) hydrogel, which we are using in other studies, was employed here a tissue simulant for the 3-D investigation of cell differentiation. Gtn-HPA 2% was cross-linked with 0.1 U/ml horseradish peroxidase (HRP) and 1.2 mM hydrogen peroxide (H_2O_2). Gtn-HPA was prepared by dissolving 2% Gtn-HPA polymers into 50% of DPBS and 50% of cell-loaded expansion medium (Dulbecco's Modified Eagle Medium-low glucose, DMEM-LG; Invitrogen). The polymer cross-linking was initiated with 0.1U/ml horseradish peroxidase (HRP; Wako Chemical, USA) and 1.2mM H_2O_2 (Sigma-Aldrich) (27). MSCs in expansion medium were added to the Gtn-HPA prior to the gelation

process, at a cell density of 1×10^5 cells/ml. In the control groups, cells received no treatment.

Extracorporeal Shock Wave Treatment

MSC's suspension was centrifuged in 15 ml tube then placed into a distilled water chamber as a conducting medium for shock wave application. SW apparatus installation was maintained to centralize the focal distance from the projectile to the cell pellets. Focused shock waves of different energy flux was delivered using focused shock wave apparatus, with energy flux1 (ESW1: 0.1mJ/mm^2 x 500 impulses), and energy flux2 (ESW2: 0.4mJ/mm^2 x 500) all impulses delivered be at 8 Hz. Cells were re-suspended to be seeded in the gel construct.

Mesenchymal Stem Cell Viability

Viability test was performed 24 hours post-gelation using the Live/Dead assay. Viability/cytotoxicity kit was used (Molecular Probes, Invitrogen) with calcein acetoxymethl easter (Calcein AM) to bind the live cells and ethidium homodimer-1 (EthD-1) to bind the dead cells at the final concentration of 2 μM and 4μM, respectively. After adding the reagents, gels were incubated for 45 min at 37℃. Afterward, gels were washed by adding 3 ml of DPBS to replace the reagents for 30 min. The cells within the gel construct were imaged by fluorescent microscope (Olympus BX60, Japan) for live and dead cells count.

Differentiation Assay

Cells in monolayer and seeded into Gtn-HPA were grown in osteogenic medium for 21 days; gel construct were fixed using 4% PFA for 2 days. Cryosections of each gel construct underwent different staining procedures. Staining of Von Kossa and Alizarin Red was performed to microscopically examine mineralization within the gels.

Effects of Shock Wave-Conditioned Medium MSC Proliferation

Second passage MSCs harvested from same animal were collected at 80-90% confluence; cells were divided into designated groups then seeded into 6-well plates with cell density of 2000 cells/ cm^2.

Group 1	MSC controls; non-shocked cells in normal expansion medium
Group 2	MSCs expanded under a conditioned medium extracted from shock wave-treated cells
Group 3	SW-treated cells kept in their medium

At Day 1, as control group, Group 1 received 3 ml of expansion medium DMEM-LG containing 10% fetal bovine serum (FBS; Invitrogen), 1% penicillin/streptomycin (Invitrogen),. Group 2 received 3 ml of shock wave conditioned medium that was extracted from shock wave treated cells (500 impulses, EFD 0.4 mj/mm^2). Group 3 MSCs treated with SW (500 impulses, EFD 0.4 mJ/mm^2) as described in section. All groups were incubated at 37C with 5% CO_2. Medium was half changed every other day.

At day 3, light microscopic images were taken for cells count. At day 6, cells were fixed by 4% paraformaldehyde at 4C overnight, followed by nuclei staining with 4'6-diamidino-2-phenylindole dihydrochloride (DAPI, Invitrogen Molecular Probes, USA). Fluorescent microscopic (Olympus BX60, Japan) images were taken for cell counting.

Effect of Shock Wave Treatment on the Osteogenic Potential of MSCs

To examine the effect of SW on bMSC stemness, similar methodology as the above experiment (Groups 1 and 3) with cell density of 4000 cells/cm^2. A surface antigen, CD105, known to be expressed by stem cells was evaluated by immunohistochemistry.

Group 1 (control) was MSCs and Group 2 was shock wave-treated MSCs. At days 0 and 7, both groups underwent a fluorescence staining using CD105 (1/100 dilution, ab156756, Abcam, USA) and counter-staining using nuclei staining with 4'6-diamidino-2-phenylindole dihydrochloride (DAPI, Invitrogen Molecular Probes, USA). Fluorescent microscopic imaging was performed at day 0 and 7 with a 10x-objective.

Osteogenic differentiation of bMSC in monolayer, identification and quantification

To identify and quantify the osteogenic differentiation of 3 groups of MSCs in monolayer, MSCs were seeded in chamber glass slides (Nunc™ Lab-Tek™ chamber slide system, Thermo Scientific, USA) with the density of 2000 cells/cm^2 per chamber. Group1 contained MSCs with normal expansion medium; Group 2 was shock wave-treated MSCs with its SW medium that was replaced by osteogenic medium after 24 hours; and Group 3 was MSCs with normal expansion medium that was replaced by osteogenic medium after 24 hours as well. Expansion medium was replaced every other day for Group 1, for Groups 2 and 3, osteogenic medium was replaced every 3 days. At day 14, cells were rinsed with PBS then fixed with 4% PFA at 4C for 30 minutes. Rinses with PBS followed by ddH2O were performed for all cultures followed by 40mM alizarin red staining (Wako Pure Chemical Industries, Ltd.) for 3 minutes at room temperature; alizarin red staining reveals divalent cations, including calcium. Cultures were then thoroughly rinsed with ddH2O and PBS to remove non-specific staining. Microscopic imaging was performed then the quantification process carried out. CPC (cetylpyridinium chloride) (Wako Pure Chemical Industries, Ltd) dissolved in 10 mM sodium phosphate buffer (pH 7.0) was added to the cultures for 1 hour at room temperature. AR-S extracts were diluted 10 times in 10% CPC solution. The AR-S content of each sample was quantified using plate-reader at absorbance of 562nm. Cell preparations were also stained for calcium using von Kossa stain.

Results

Stem Cell Markers

The MSCs displayed positive staining for the surface antigen, CD105, routinely used as a stem cell marker (Fig. 1 A-C). This positive staining was found on day 1 as control after the MSCs were treated with SW2 (500 impulse of 0.4 mJ/mm^2). At day 7, both control and SW2 treated cells stained negative for CD105, which indicates their differentiation into select cells.

Effect of SW2–Conditioned Medium on MSCs Proliferation in Monolayer

Exposure of MSCs in monolayer to SW-conditioned medium increased the proliferation of MSCs compared to the control. All groups substantially increased in cell numbers by more than 3- fold from 3-6 days in culture (Fig. 2). Two-factor ANOVA demonstrated statistically significant effect of groups ($p=0.036$; power= 0.63) and time in culture ($p < 0.0001$; power=1) on the number of MSCs. Fisher's PLSD *post hoc* test showed that there was a statistically significant difference between the number of MSCs treated with SW-conditioned medium (Group 2) and the MSCs in the control expansion medium (Group 1; $p=0.01$). There was no statistically significant difference between Group 2 and 3.

After 3 days there was a statistical significant effect of SW-conditioned medium on the number of cells in culture (an increase of 25%; one factor ANOVA, p=0.024; power= 0.64) After 6 days, there was around 20 % increase in proliferation of the MSCs in SW-conditioned medium but was not statistically significant by one-factor ANOVA, (p=0.06, power 0.44)

At 3 days, there was no statistically significant effect of SW treatment when comparing the proliferation of non- shocked MSCs grown in SW-conditioned medium and shocked cells grown in their own medium (p=0.3%; power 0.13). There was no statistical difference between these 2 groups at 6 days (one-factor ANOVA, p=0.1 %, power=0.25).

MSC Osteogenic Differentiation

Examining the calcium formation reflecting osteogenic differentiation in monolayer, SW2-treatd cells grown with osteogenic medium showed significantly higher calcium formation compared to MSCs grown in expansion medium (Fig. 3). Osteogenic medium-SW2 cells showed a greater amount of calcium formation than non-shocked cells in osteogenic medium (Fig. 3), but this finding was not statistically significant.

Gtn-HPA 2% hydrogels supported the differentiation of MSCs into osteogenic-like cells (Fig 4A-F). Gtn-HPA cross-linking supported the intercellular communication for mineral formation within different layers of the gel construct.

Discussion

This set of experiments demonstrated that MSC treated with ESWs release a paracrine factor that stimulates the proliferation of MSCs. ESW treatment did not effect the osteogenic differentiation of MSCs.

Specific Aim #3

Task 4. Evaluation of the cartilage repair induced by ESW-stimulated periosteum in a goat model

4.a. ESW stimulation of the periosteum in the proximal tibia

4.b. Harvest of the ESW-treated periosteum and sham controls after 4 days, and implantation into chondral defects in the trochlear groove of the same animals

4.c. Sacrifice of the goats 16 weeks post-implantation, and processing of tissue for histological evaluation.

4.d. Histomorphometric evaluation of the cartilage repair
This aim is to be achieved in future experiments.

III. KEY RESEARCH ACCOMPLISHMENTS

- ESWs causes MSCs to release a mitogenic paracrine factor.
- ESW treatment does not affect the osteogenic differentiation of MSCs.

IV. REPORTABLE OUTCOMES

None.

V. CONCLUSIONS

These results demonstrate the range of tissue responses that can be induced by ESWs.

VI. REFERENCES

None

VII. APPENDICES None

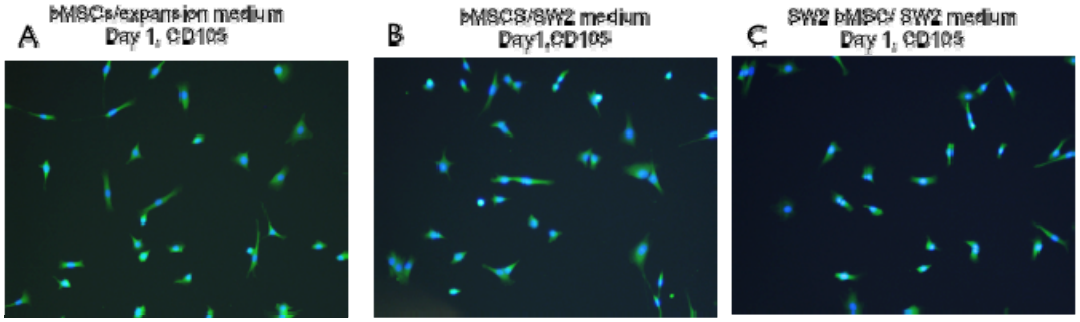

Fig.1. Fluorescence Images for CD105 at day 1, A) bMSC in expansion medium (control) B) bMSCs in SW conditioned medium C) SW treated bMSC in SW conditioned medium (n=4).

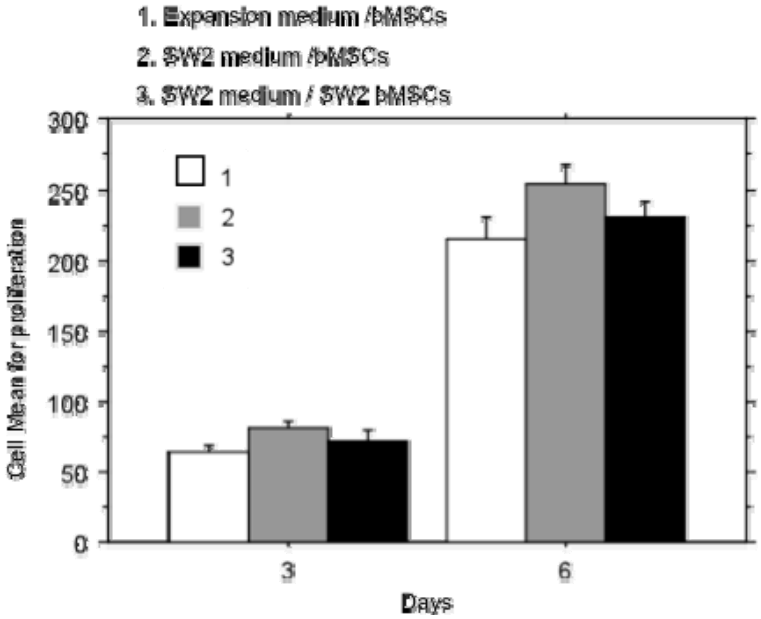

Fig. 2. Effect of SW2 –conditioned medium on bMSCs proliferation in monolayer after 3 and 6 days.

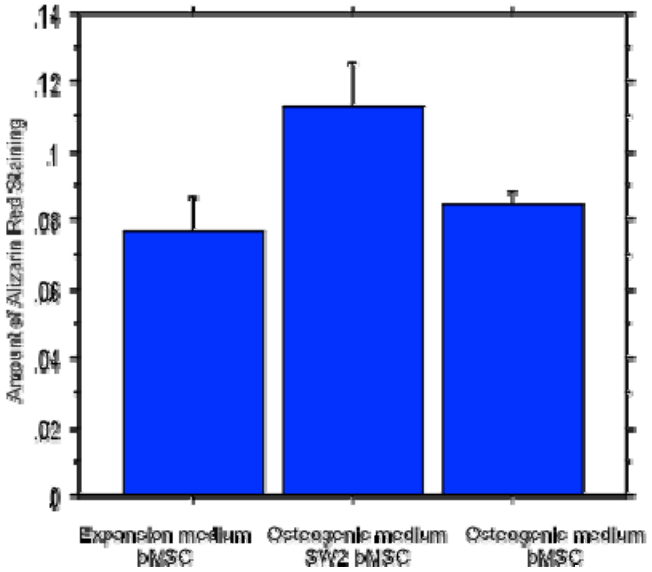

Fig. 3. Effect of shock waves on bMSCs osteogenic differentiation in monolayer.

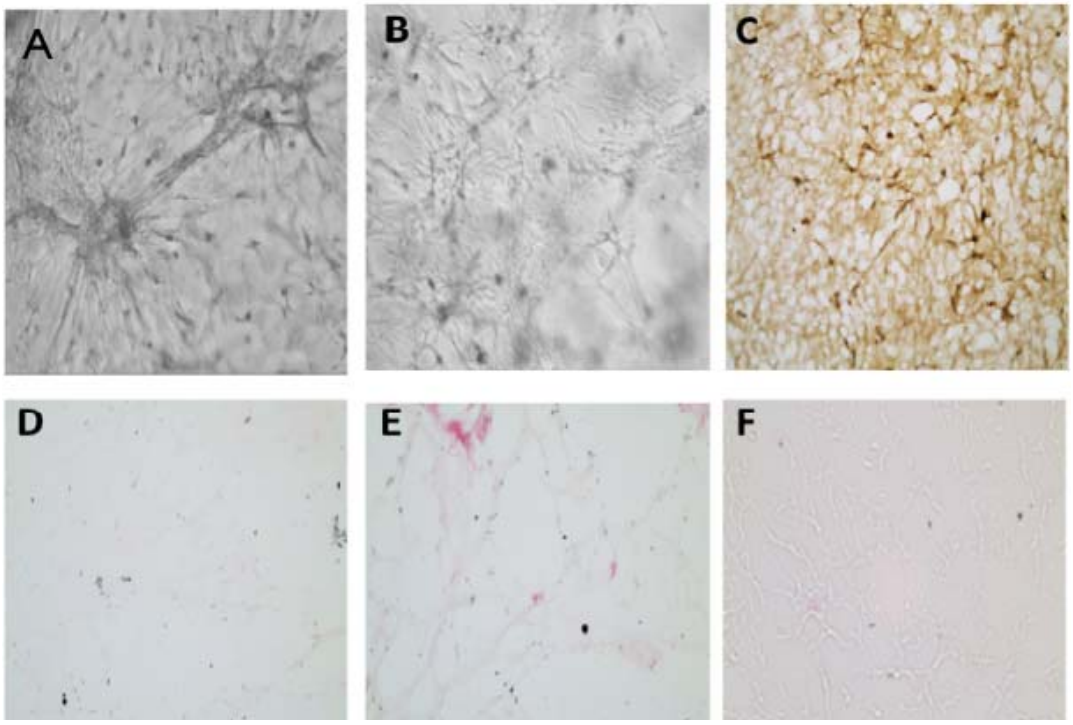

Fig. 4. (A,B) bMSCs encapsulated within Gtn-HPA undergo osteogenic differentiation (C) Alizarin Red staining showing calcium formation within the Gtn-HPA after 21 days. (D-F) Von Kossa staining showing mineralization within the Gtn-HPA gel.